Issued August 3, 1915.

HAWAII AGRICULTURAL EXPERIMENT STATION,

J. M. WESTGATE, Agronomist in Charge.

Bulletin No. 39.

THE BIOCHEMICAL DECOMPOSITION OF NITROGENOUS SUBSTANCES IN SOILS.

BY

W. P. KELLEY,

Chemist.

UNDER THE SUPERVISION OF

STATES RELATIONS SERVICE.

U. S. DEPARTMENT OF AGRICULTURE.

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HAWAII AGRICULTURAL EXPERIMENT STATION, HONOLULU.

[Under the supervision of A. C. TRUE, Director of the States Relations Service, United States Department of Agriculture.]

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[1] Resigned October 27, 1914.

LETTER OF TRANSMITTAL.

Hawaii Agricultural Experiment Station,
Honolulu, Hawaii, September 14, 1914.

Sir: I have the honor to submit herewith and recommend for publication as Bulletin No. 39 of the Hawaii Experiment Station a paper on The Biochemical Decomposition of Nitrogenous Substances in Soils, by W. P. Kelley, chemist. More exact information is needed on the sources from which plants draw nitrogen for their growth. This bulletin contains the results of a study of the percentages of ammonia derived from the bacterial decomposition of various organic nitrogenous substances in soils. In six out of the eight substances used in this study the basic diamino acid nitrogen was found to be more readily converted into ammonia than was the nitrogen of other groups.

Respectfully,

E. V. Wilcox,
Special Agent in Charge.

Dr. A. C. True,
Director States Relations Service,
U. S. Department of Agriculture, Washington, D. C.

Publication recommended.
A. C. True, *Director.*

Publication authorized.
D. F. Houston,
Secretary of Agriculture.

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INTRODUCTION.

The chemical changes produced in the nitrogenous substances of soils by bacteria are of great importance. As is well known, the end products are ammonia, nitrate, and free nitrogen, but the intermediate steps of the change, which are probably of great importance, are only imperfectly understood. It appears that proteins undergo progressive decomposition in soils similar to that which takes place in animal digestion, and it is highly probable that ammonification is preceded by hydrolysis. From the investigations of Schreiner et al.[1] it has been shown that small amounts of protein and nucleoprotein cleavage products are widely distributed in soils; and Löhnis and Green[2] recently found from ammonification experiments with flesh meal that in the early stages of the action greater amounts of ammonia were obtained by distilling a 1 per cent hydrochloric acid extract with caustic soda than with magnesia. They held that the caustic soda decomposed soluble hydrolytic products (amino acids) split off by the bacteria. At a later stage, when the amino acids had presumably been more completely decomposed, the yields of ammonia by the two methods were more nearly equal.

It is probable that not all of the nitrogen in a given protein is equally susceptible to ammonification[3] and that different proteins undergo decomposition at different rates, even when all other conditions are equal. In ammonification experiments, for example, it is seldom that more than from 50 to 60 per cent of the nitrogen added is recovered as ammonia, and different nitrogenous substances yield ammonia at greatly different rates. As is well known, the main portion of the nitrogen in soils occurs in organic forms and has previously existed as vegetable protein. After the organic matter of soils has been acted upon by bacteria for a time a residue, rich in nitrogen, and commonly called humus, is left, which undergoes further decomposition at a very slow rate. Moreover, the rate of formation of ammonia from humus appears to depend to some extent on the conditions under which the humus was itself formed. Generally acid

[1] U. S. Dept. Agr., Bur. Soils Buls. 53, 74, 80, 83, 88.
[2] Centbl. Bakt. [etc.], 2. Abt., 37 (1913), pp. 534-562.
[3] See Jodidi, Iowa Sta. Research Bul. 9 (1912).

humus, or humus formed under anaerobic conditions, is thought to decompose more slowly than neutral humus. Thus it seems that conditions arise during the course of bacterial action which tend to check the process, or else some of the constituents of proteins are more difficult of hydrolysis and decomposition than others.

Previous investigations on proteins throw much light on this subject. It is known that proteins are composed of a number of amino acids and that different proteins undergo hydrolysis by acids, alkalis, and enzyms at different rates, yielding varying amounts of the several amino constituents. From limited study on the subject it seems that bacterial and enzymatic action bring about similar hydrolysis of proteins, with the important difference that the products of hydrolysis are subject to decomposition by bacteria. During the course of such action certain amino acids are split off more rapidly than others. Abderhalden and Reinbold [1] found, for example, that in the tryptic digestion of edestin from cotton seed, 97.6 per cent of the tyrosin had been split off at the end of 2 days, while only 7.4 per cent of the glutaminic acid had been hydrolyzed.

From previous work in this laboratory [2] it has been shown that Hawaiian soils contain relatively greater amounts of amid and smaller amounts of basic (diamino acid) nitrogen than the vegetable proteins. From this it has been suggested that a study of the group changes produced under the action of bacteria might throw important light on both the availability of, and the nature of bacterial action on, nitrogenous fertilizers. Various factors, such as the degree of aeration, the acidity of the medium, the carbohydrates present, the synthesis of proteins in the body cells of the bacteria, the absorption of organic nitrogen compounds in varying degrees by plants, etc., so complicate the problem as to render very difficult an interpretation of the chemistry of bacterial action in soils. It has been suggested, however, that by also studying the rates of decomposition under varying conditions some light might be thrown on this question.

AMMONIFICATION UNDER VARYING CONDITIONS.

SERIES I—AMMONIFICATION IN SILICA SAND.

The materials used in this investigation contained the following percentages of nitrogen: Casein (Eimer and Amend), 12.40 per cent; dried blood, 13.29 per cent; soy bean cake meal, 8.28 per cent; cottonseed meal, 5.10 per cent; linseed meal, 5 per cent.

In order to insure maximum aeration the first series of tests was conducted with the use of silica sand. One gram each of the above nitrogenous materials and one gram of calcium carbonate were thoroughly mixed with 100-gram portions of sand in tumblers. Ten cubic

[1] Ztschr. Physiol. Chem., 46 (1905), pp. 159-175.

[2] Hawaii Sta. Bul. 33 (1914).

centimeters of an infusion, made by shaking for ten minutes 200 grams of fresh soil, taken from the citrus orchard of this station, with 250 cubic centimeters of sterile water was then added to each beaker and thoroughly mixed with the contents. The beakers were covered with watch glasses and the mixtures incubated at 28° C. The ammonia was determined at intervals, as shown in the table, by three different methods. One portion was distilled directly with magnesia; three other portions were first shaken with 250 cubic centimeters of 1 per cent hydrochloric acid, allowed to stand for an hour, and then filtered. An aliquot of one of these, corresponding to 75 per cent of the material used, was made alkaline with magnesia and distilled, while aliquots of the other two portions were distilled with caustic soda. The results obtained were as follows:

Amount of ammonia formed in silica sand from different substances.

Period of incubation.	Method of determination.	Nitrogen as ammonia from—				
		Casein.	Dried blood.	Soy bean cake meal.	Cotton-seed meal.	Linseed meal.
		Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*
2 days	Distilled directly with MgO	49.0	4.5	13.4	6.2	7.3
	HCl solution distilled with MgO	47.2	2.8	12.0	4.8	5.6
	HCl solution distilled with NaOH	44.4	4.2	9.9	4.3	6.4
		42.0	4.2	10.3	4.1	5.6
4 days	Distilled directly with MgO	52.1	14.3	27.9	12.7	16.2
	HCl solution distilled with MgO	44.8	15.0	20.5	12.3	13.1
	HCl solution distilled with NaOH	44.8	15.9	22.4	12.5	14.9
		41.2	15.0	22.6	11.6	14.7
6 days	Distilled directly with MgO	Lost	26.0	21.7	12.9	17.9
	HCl solution distilled with MgO	41.1	24.9	20.4	10.4	14.2
	HCl solution distilled with NaOH	35.5	Lost	20.2	10.4	15.9
		34.4	29.1	20.2	12.3	16.8
9 days	HCl solution distilled with MgO	25.2	19.8	13.0	11.2	14.9
	HCl solution distilled with NaOH	24.8	23.5	19.6	9.9	14.7

The different materials were converted into ammonia at greatly different rates. Casein was the most readily ammonified, the concentration of ammonia having reached a maximum at the end of two days, when about 40 per cent of the nitrogen had been converted into ammonia. After four days the ammonia content became considerably reduced. In view of the yields of ammonia found in experiments with soil (Series II) it is probable that ammonification was active for longer than two days, but then evaporation and nitrification more than equaled ammonification. The rapidity with which casein[1] was ammonified as compared with the other materials is especially interesting.

The formation of ammonia from dried blood took place slowly during the first two days, but later the yield approached that from casein. Soy bean cake meal was ammonified considerably more

[1] See Brown, Iowa Sta. Research Bul. 11; Centbl. Bakt. [etc.], 2. Abt., 39 (1913), pp. 61–73.

rapidly during the first four days than dried blood, after which time the yields decreased; while cottonseed meal and linseed meal each gave lower yields, the highest concentration of ammonia from the former being on the fourth day and from the latter on the sixth day.

Direct distillation with magnesia after two days yielded slightly more ammonia in most instances than the other methods. Later the results by the different methods were fairly concordant. With only a few exceptions, as large amounts of ammonia were obtained by distilling the hydrochloric acid extracts with magnesia as with caustic soda.[a] The above data therefore give but little indication that hydrolysis was more rapid than ammonification.

SERIES II—AMMONIFICATION IN SOIL.

This series of experiments was conducted with the fresh heavy clay soil from which the infusions were made in the previous series. One-hundred-gram portions were each thoroughly mixed with 1 gram of the nitrogenous materials and 1 gram of calcium carbonate and placed in tumblers. Sterile water was added so as to bring the moisture up to 25 per cent. After incubating at 28° C. the ammonia was determined as in the previous series. On the ninth day the nitrate was also determined in separate portions by the phenol disulphonic acid method, and was added to the average ammonia at this period in estimating the total ammonification.

Amount of ammonia formed in soil from different substances.

Period of incubation.	Method of determination.	Nitrogen as ammonia from—				
		Casein.	Dried blood.	Soy bean cake meal.	Cotton-seed meal.	Linseed meal.
		Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*
2 days	Distilled directly with MgO	50.8	4.1	7.8	5.6	2.0
	HCl solutions distilled with MgO	38.1	2.2	5.6	4.8	1.1
	HCl solutions distilled with NaOH	40.0	4.2	8.1	4.2	4.2
		40.3	4.5	7.8	5.6	2.8
4 days	Distilled directly with MgO	69.4	24.1	21.8	11.5	11.2
	HCl solutions distilled with MgO	57.7	18.5	20.4	9.5	9.5
	HCl solutions distilled with NaOH	65.5	21.3	17.4	8.1	9.5
		60.5	21.6	19.9	10.1	10.6
6 days	Distilled directly with MgO	68.6	42.4	29.4	10.6	13.9
	HCl solutions distilled with MgO	59.1	40.9	26.3	9.2	10.9
	HCl solutions distilled with NaOH	56.6	33.6	28.3	10.6	10.9
		56.8	35.2	23.8	9.8	11.2
9 days	HCl solutions distilled with MgO	59.1	[b] 38.9	26.6	7.8	7.0
	HCl solutions distilled with NaOH	56.8	49.0	27.2	7.3	9.8
9 days	Nitrate N	4.4	7.4	7.0	6.3	4.6
9 days	Total ammonification	62.3	56.4	33.9	13.8	13.0
	Per cent of total N converted into NH_3.	50.2	42.4	40.9	27.1	26.0

[a] It is not surprising that practically as much ammonia was obtained by distilling the solutions with magnesia as with caustic soda, since the acid amids are decomposed by each alike, and only two other protein cleavage products, cystin and arginin, yield ammonia to caustic soda. Cystin occurs in very small amounts in most proteins.

[b] Not used in average.

The amounts of ammonia recovered from the different materials after two days and the relative rates of ammonification throughout bear similar relations to those of the previous series. In the cases of casein, dried blood, and soy bean cake meal, from which the largest yields of ammonia were obtained in the sand cultures, still greater amounts accumulated in the soil. The ammonification of cottonseed meal and linseed meal was more nearly equal at each period, being practically the same as that which took place in sand. The losses by evaporation were considerably less than from the sand, due probably to the high absorbing power of the clay. A small amount of nitrification took place, but it was not proportional to ammonification. The data for the total ammonification show a wide difference in the decomposition of the materials; 50.2 per cent of the nitrogen in casein was converted into ammonia, 42.4 per cent in dried blood, 40.9 per cent in soy bean cake meal, 27.1 per cent in cottonseed meal, and 26 per cent in linseed meal. Thus the availability of these materials as measured by ammonification varies greatly.

Direct distillation with magnesia, especially in those instances where comparatively large amounts of ammonia had accumulated, yielded considerably more ammonia than the distillation of hydrochloric-acid extracts; but, again, distillation of the latter with magnesia yielded as much ammonia as distillation with caustic soda. It would seem, therefore, that direct distillation with magnesia affords a truer measure of ammonification in clay soils than indirect distillation of hydrochloric-acid solutions. In all of the subsequent series reported in this bulletin the former method was used.

SERIES III—AMMONIFICATION OF EQUAL AMOUNTS OF NITROGEN.

In this series the nitrogenous materials were added so as to furnish equal amounts of nitrogen (132.9 milligrams). These and 1 gram of calcium carbonate were mixed with 100 grams of the same fresh soil. After bringing the moisture content to 25 per cent and incubating as before the yields of ammonia were as follows:

Amount of ammonia from equal amounts of nitrogen.

[Average of 2 samples.]

Period of incubation.	Nitrogen as ammonia from—				
	Casein, 1.072 gm.	Dried blood, 1.000 gm.	Soy bean cake meal, 1.605 gm.	Cotton-seed meal, 2.606 gm.	Linseed meal, 2.658 gm.
	Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*
2 days	58.3	4.0	14.9	17.3	3.5
4 days	74.9	38.5	47.1	37.9	32.9
6 days	75.7	59.6	64.8	42.6	39.9
9 days	75.2	65.6	58.6	40.3	46.0
Per cent of total N converted into NH_3	56.9	49.3	48.7	32.0	34.6

The foregoing data show that when equal amounts oi nitrogen were added, the amounts of ammonia formed still varied considerably. Casein was far more readily decomposed during the early stages of the action than the other materials. At the end of 2 days 58.3 milligrams of casein nitrogen had been ammonified, as contrasted with 4 milligrams in dried blood, 14.9 milligrams in soy bean cake meal, 17.3 milligrams in cottonseed meal, and 3.5 milligrams in linseed meal. Later the yields became more nearly equal and showed much less variation than when equal weights of the materials were added (see Series II). The maximum percentages of ammonia formed from the different materials, not allowing for evaporation and nitrification, the latter of which was small, ranged from 56.9 per cent from casein to 32 per cent from cottonseed meal.

SERIES IV—AMMONIFICATION IN SOIL UNDER ANAEROBIC CONDITIONS.

In order to measure the rates of ammonification under anaerobic conditions the same soil was used and sufficient sterile water added to insure complete submergence. Equal amounts of nitrogen (132.9 milligrams) and 1 gram of calcium carbonate were mixed with 100-gram portions of soil as in the preceding series.

Amount of ammonia in soil under anaerobic conditions.

[Average of 2 samples.]

Period of incubation.	Nitrogen as ammonia from—				
	Casein 1.072 gm.	Dried blood 1 gm.	Soy bean cake meal 1.606 gm.	Cotton-seed meal 2.605 gm.	Linseed meal 2.658 gm.
	Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*
2 days	8.5	2.0	3.5	6.3	2.0
4 days	47.3	6.6	9.1	8.2	3.5
6 days	62.4	13.7	14.2	9.4	7.3
9 days	70.7	16.3	18.6	11.4	9.2
Per cent of total N converted into NH_3	53.2	12.3	14.0	8.5	6.9

The above data show that under anaerobic conditions active ammonification of casein did not begin until after two days' standing, but it then was approximately as rapid as under aerobic conditions. With dried blood, soy bean cake meal, cottonseed meal, and linseed meal ammonification took place at greatly reduced rates throughout the experimental period. The percentages of the total nitrogen converted into ammonia were as follows: Casein 53.2 per cent, dried blood 12.3 per cent, soy bean cake meal 14 per cent, cottonseed meal 8.5 per cent, and linseed meal 6.9 per cent. By comparing these data with the preceding it will be seen that anaerobic conditions greatly retarded the formation of ammonia from all the

materials except casein. As is well known, a wide range of organisms, including bacteria and fungi, have the power of splitting ammonia from organic materials. Some of these are aerobic, some anaerobic, and others facultative anaerobic. When anaerobic conditions are brought about, the formation of ammonia has usually been found to be considerably less than under aerobic conditions.

SERIES V—AMMONIFICATION WITH EQUAL AMOUNTS OF NITROGENOUS AND NONNITROGENOUS MATERIALS.

In order to make the conditions for bacterial action more nearly equal, the different materials were added so as to furnish equal amounts of nitrogen (132.9 milligrams), and the inequalities in the amounts of nonnitrogenous materials were balanced by the addition of the proper amounts of cornstarch. One gram of calcium carbonate and 100-gram portions of the above soil were used. Optimum moisture conditions were provided and the same methods employed as in the previous series.

Amount of ammonia formed, with equal amounts of nitrogenous and nonnitrogenous materials.

[Average of 2 samples.]

Period of incubation.	Nitrogen as ammonia from—				
	Casein 1.072 gm., starch 1.586 gm.	Dried blood 1gm., starch 1.658 gm.	Soy bean cake meal 1.605 gm., starch 1.053 gm.	Cottonseed meal 2.606 gm., starch 0.052 gm.	Linseed meal 2.658 gm.
	Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*
2 days	31.0	0.3	4.4	10.3	2.4
4 days	30.9	3.3	20.1	34.8	25.8
6 days	35.8	10.4	33.5	42.6	36.2
9 days	41.8	25.2	45.4	45.2	45.3
Per cent of total N converted into NH_3	31.4	18.9	34.1	34.0	34.1

By comparing the above with Series III it will be seen that the addition of starch materially influenced the accumulation of ammonia. By adding 1.586 grams of starch the ammonification of casein was reduced practically 50 per cent throughout the nine days. The effects on the ammonification of dried blood were still more marked. At the end of two days practically no ammonia had appeared and the depressing effect was observed throughout the experimental period; after nine days only 18.9 per cent of the nitrogen added as dried blood occurred in the form of ammonia. A similar, although less marked depressing effect, was observed with soy bean cake meal, but it must be remembered that 0.605 gram less starch was added than with the dried blood. The final yields of ammonia from soy

bean cake meal, cottonseed meal, and linseed meal were almost the same, being equal to 34.1 per cent of the total nitrogen added. Thus it is shown, in common with the findings of others, that the carbon nitrogen ratio may greatly affect the accumulation of ammonia in soils.

The low yields of ammonia in the presence of carbohydrates have been attributed to stimulation of the ammonia-consuming organisms, whereby ammonia is reconverted into organic forms, and to the formation of metabolic by-products, probably of an acid nature, which exercise an inhibitory influence on ammonification; but, as shown by Lipman et al.,[1] the depressing effect of carbohydrates can not be prevented by adding calcium carbonate. It is probable that the energy derived by the ammonifying organisms themselves from the nonnitrogenous matter is of considerable importance. It is true these organisms can satisfy their energy requirements from amino compounds of various sorts, but it does not follow that a part of it could not be derived more advantageously from carbohydrates.[2] The fact that bacteria split off ammonia from nitrogenous substances, thus eliminating a portion of the nitrogen present, is in itself evidence of their demand for nonnitrogenous matter.

It should be borne in mind that the nonnitrogenous constituents of the above materials were made up of different chemical compounds including fats and carbohydrates. The exact effect of fats is not known, but different carbohydrates produce widely different effects. In general, soluble carbohydrates more markedly depress ammonification than insoluble forms.[3] The nature of nitrogenous constituents in these materials also differs considerably, and the rate at which they undergo hydrolysis, with the exception of casein, has not been extensively studied. As will be shown later, the products of acid hydrolysis vary considerably. Since hydrolysis is probably essential as preliminary to ammonification [4] any differences in the rates of hydrolysis would probably be reflected in the rates of ammonification.

SERIES VI—AMMONIFICATION WITH VARYING AMOUNTS OF CASEIN.

In the preceding series the yields of ammonia from practically the same amounts of casein varied from 50.2 per cent to 56.9 per cent of the total nitrogen added. In the following series the concentration of casein was varied and the same amounts of soil and calcium carbonate were used throughout. For the purpose of reducing evapo-

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[1] New Jersey Stas. Rpt. 1911, pp. 193–212.

[2] See J. G. Lipman et al., New Jersey Stas. Rpt. 1909, pp. 166–169.

[3] See Lipman et al., New Jersey Stas. Rpt. 1911, pp. 193–212.

[4] The fact that peptone has frequently been found to ammonify more rapidly than dried blood or cottonseed meal may be due in part to its being a partially hydrolyzed substance.

ration losses, 1 gram of monomagnesium phosphate was also added, as recommended by Löhnis and Green.[1] After bringing the moisture to about 25 per cent and incubating at 28° C. for four days, the ammonia was determined as before with the following results:

Amount of ammonia formed from varying quantities of casein.

[Average of 2 samples.]

Amount of casein added.	N found as ammonia.	Per cent of total N recovered as NH_3.	Amount of casein added.	N found as ammonia.	Per cent of total N recovered as NH_3.
Gm.	*Mg.*		*Gm.*	*Mg.*	
0.2	12.0	48.4	1.5	[1] 111.7	60.1
.4	25.5	51.4	2.0	151.0	60.9
.6	43.5	58.2	2.5	192.3	62.0
.8	58.2	58.7	3.0	244.1	65.9
1.0	70.7	57.0			

[1] 1 sample only.

The percentages of total nitrogen recovered as ammonia increased as the amount of casein increased, varying from 48.4 per cent with 0.2 gram to 65.9 per cent with 3 grams (see fig. 1). Loss of ammonia by evaporation was not important, since the percentage yields were greatest where the greatest concentration of ammonia occurred. Since almost no nitrification took place in any instance, it seems reasonable to believe that, as the amount of casein added is increased, a decreasing percentage of the total nitrogen present would be consumed by the bacteria and consequently higher percentage yields of ammonia be obtained. The yield from 1 gram (57 per cent) agrees closely with that recovered in preceding series.

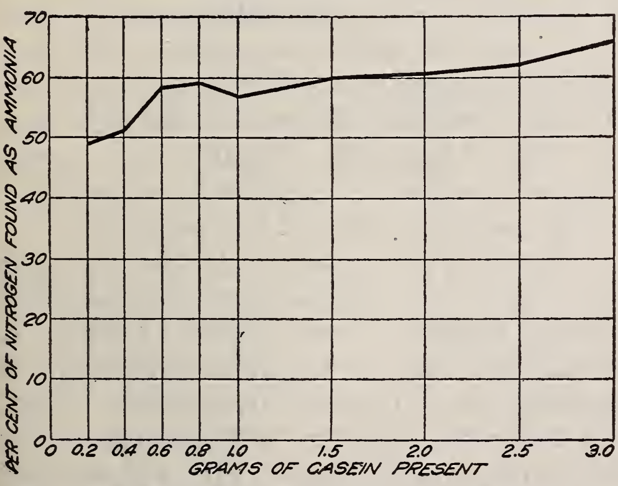

FIG. 1.—Diagram showing the ammonification of different amounts of casein.

SERIES VII—AMMONIFICATION OF CASEIN DURING DIFFERENT LENGTHS OF TIME.

This series was begun at the same time as Series VI, using the same soil. One gram each of casein, calcium carbonate, and magnesium phosphate was mixed with 100-gram portions of soil, sterile water

[1] Loc. cit.

added, and the mixture incubated as before. After four and eight days, respectively, an additional gram of casein was added in certain instances and the ammonia and nitrate determined, as shown in the following table:

Amount of nitrogen recovered from casein.

[Average of 2 samples.]

Amount of casein and period of incubation.	N recovered as NH_3.	N recovered as NO_3.	Per cent of total N converted into NH_3.
	Mg.	*Mg.*	
1 gram 4 days	72.1	....	57.3
1 gram 8 days	74.2	....	59.8
1 gram 12 days	60.2	12.5	58.6
1 gram 4 days+1 gram 4 days additional	150.9	....	60.8
1 gram 4 days+1 gram 8 days additional	138.9	6.6	57.1
1 gram 4 days+1 gram 4 days additional+1 gram 4 days	226.8	3.3	61.8

Practically the same amounts of ammonia were formed from a given amount of casein in four days as in longer periods. The yields from 1 gram were 57.3 per cent in four days, 59.8 per cent in eight days, and 58.6 per cent in twelve days. By adding another gram on the fourth day and allowing the action to continue four days longer 60.8 per cent of the total nitrogen was converted into ammonia. The portions treated in the same way but allowed to stand eight days longer gave 57.1 per cent yield of ammonia. Finally when 1 gram was added at the beginning and after four and eight days, respectively, 61.8 per cent of the total nitrogen was converted into ammonia.

The above data show, therefore, in common with the preceding series, that increasing percentages of the total nitrogen were converted into ammonia when increasing amounts of casein up to 3 grams were acted upon, but whether this fact was due to partial suppression of the nonammonifying organisms can not be positively stated. There is evidence, however, that under the conditions of these experiments the organisms feed on the organic nitrogen of casein rather than on the ammonia after it has been formed.

SERIES VIII—AMMONIFICATION OF CASEIN IN SILICA SAND.

The amounts of ammonia recovered from casein in the preceding experiments usually did not exceed 60 per cent of the nitrogen added, and reached a maximum point by the fourth day. In order to throw further light on this subject a series of experiments was carried out with silica sand, provision being made for absorbing whatever ammonia was volatilized. The decomposition took place in closely stoppered bottles through which a slow current of air was drawn by means of a suction pump. The current of air was first drawn through sulphuric acid to remove traces of ammonia and after passing through

the bottles was again drawn through a solution of sulphuric acid. Casein prepared by Hammarsten was employed. This was further purified by first dissolving in tenth-normal sodium hydroxid solution, then precipitating with 1 per cent acetic acid, filtering, and thoroughly washing with alcohol and ether. After drying in vacuum over sulphuric acid, the product was found to contain only 13.50 per cent nitrogen, showing that considerable impurities still remained. (Pure casein contains 15.62 per cent N.)

Five grams of the casein was mixed with 500 grams of silica sand and optimum moisture brought about by adding 50 cubic centimeters of a soil infusion prepared as in Series I. After incubating at 28° C. for one week the contents of the bottles were acidified with 1 per cent acetic acid, thoroughly shaken, filtered, and washed. The residue was then extracted with tenth-normal sodium hydroxid and the alkaline solutions acidified with 1 per cent acetic acid. No precipitate was formed and the solutions were combined with those above. Ammonia was determined in the solutions, sand residues, and the sulphuric acid by distilling with an excess of magnesia.

Total amount of ammonia formed from casein in silica sand.

Determination No.	N as NH_3 soluble in acetic acid.	N as NH_3 in the residue.	N as NH_3 volatilized.	Total yield of NH_3.	Per cent of total N converted into ammonia.
	Mg.	*Mg.*	*Mg.*	*Mg.*	
1	271.6	96.6	62.6	430.8	63.8
2	273.0	96.6	76.4	446.0	66.1
3	324.8	55.7	43.4	423.9	62.8

A slightly higher yield of ammonia was obtained than previously, the average being 64.2 per cent of the total nitrogen. Since no casein could be precipitated from sodium hydrate solutions after bacterial action, the conclusion that the entire amount of casein added had undergone hydrolysis is justified. The nature of the undetermined balance remains to be determined.

SERIES IX—AMMONIFICATION AND HYDROLYSIS OF CASEIN.

The purpose of this series was to study the relations between the rates of ammonification and hydrolysis of casein. A stock solution of casein purified as in the previous series was prepared by dissolving 15 grams in 150 cubic centimeters of tenth-normal sodium hydroxid, then diluting to 2,100 cubic centimeters. One hundred cubic centimeter portions were placed in 300 cubic centimeter Erlenmeyer flasks and 10 cubic centimeters of soil infusion added. After shaking, the flasks were loosely stoppered with cotton plugs and incubated at 28° C. The rate of hydrolysis was measured by precipitating the

unhydrolyzed casein at intervals with 1 per cent acetic acid, then making total nitrogen determinations in the precipitates as recommended by Walters.[1] Ammonia was determined in the filtrates by distilling with an excess of magnesia. In order to have a check on autohydrolysis, which is known to take place in solutions of casein, a number of the flasks were set aside without the addition of infusions and bacterial action prevented by the addition of 0.2 cubic centimeter of toluol. The casein was precipitated from these at intervals by adding 20 cubic centimeters of 1 per cent acetic acid and the nitrogen determined as above. The data showing autohydrolysis will be submitted first.

Autohydrolysis of casein in solution.

[Average of 2 samples.]

Period of incubation.	N present.	N precipitated.	N hydrolyzed.	Per cent of N hydrolyzed.
	Mg.	*Mg.*	*Mg.*	
1 hour	94.3	90.4	3.9	4.13
3 days	94.3	89.6	4.7	4.96
9 days	94.3	87.6	6.4	6.79

The above data show that in one hour's time 4.13 per cent of the nitrogen underwent autohydrolysis, and this was increased upon standing for 9 days to 6.79 per cent.

The effects of bacterial action on the ammonification and hydrolysis of casein are shown as follows:

Some results of bacterial action on casein.

[Average of 2 samples.]

Period of incubation.	N added.	N found as NH_3.	N precipitated.	Per cent of N ammonified.	Per cent of N hydrolyzed.
	Mg.	*Mg.*	*Mg.*		
1 day	94.3	0.0	90.4	0.00	4.13
3 days	94.3	.6	91.1	.64	3.39
4 days	94.3	2.5	75.9	2.65	19.51
5 days	94.3	9.7	29.6	10.28	68.61
7 days	94.3	56.1	13.8	59.53	85.36
9 days	94.3	58.7	17.6	62.25	81.32

Active ammonification set in after the fourth day and reached a practical maximum on the seventh day, when 59.53 per cent of the nitrogen had been converted into ammonia. Active hydrolysis set in after the third day and was completed by the seventh day. By this time the solutions had become quite opalescent, due to the abundance of cells of bacteria and fungi, and no precipitate was

[1] Jour. Biol. Chem., 11 (1912), pp. 267–305.

formed upon acidifying with acetic acid. The nitrogen recorded as precipitated on the seventh and ninth days, therefore, was not in the form of casein but was contained in the bacterial cells that were held up by the filter paper.

From the foregoing it is apparent that bacterial hydrolysis of casein precedes ammonification and that the former takes place considerably more rapidly than the latter.

EFFECTS OF BACTERIAL ACTION ON DIFFERENT GROUPS OF NITROGEN COMPOUNDS.

In the preceding experiments maximum ammonification usually took place in from four to six days. Of the substances tested casein was the most completely ammonified, but as stated above, usually not more than 60 per cent of the nitrogen was converted into ammonia.

The following experiments were made for the purpose of studying the effects of bacterial action on the different groups of organic nitrogen compounds. Two gram portions of the substances were mixed with 100 grams of silica sand, soil infusions added and incubated for definite periods. Then the sand mixtures were transferred to 1,000 cubic centimeter Kjeldahl flasks, 400 cubic centimeters of hydrochloric acid added, and the whole boiled under reflux condensers for 10 hours. After filtering and washing the residue with hot water, the filtrates were diluted to 1,000 cubic centimeters and aliquots used in the determination of the amid, basic, and nonbasic groups of nitrogen compounds, employing the same methods as were used in previous work on the organic nitrogen of Hawaiian soils.[1]

In every case the residues left after filtration were practically free from nitrogen, showing that all the nitrogen present went into solution, but a smaller amount was generally found than occurred in the original materials. This was probably due to the loss of ammonia by volatilization during the course of bacterial action, and to the decomposition of nitrates, and therefore, will be considered as having been converted into ammonia. It is possible, however, that some denitrification also took place. The length of time that the different materials were exposed to bacterial action varied, the purpose being to allow decomposition to continue no longer than was necessary to insure vigorous ammonification. Ammonia was determined in separate portions by direct distillation with magnesia. The original materials were also subjected to acid hydrolysis and the group determinations made as above. All determinations were made in duplicate.

The materials studied include casein, dried blood, soy bean cake meal, cottonseed meal, and linseed meal whose nitrogen contents

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[1] Hawaii Sta. Bul. 33 (1914).

have already been given. Coconut meal, containing 3.30 per cent nitrogen, globulin from cottonseed meal, containing 16.38 per cent nitrogen, and zein from maize, containing 14.03 per cent nitrogen, were also used. Neither the globulin nor the zein was entirely pure, as is shown by the nitrogen content.

CASEIN.

Nitrogen content of casein and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	3.93	....	31.96	....	....
Amid N	1.54	1.10	12.43	8.88	28.57	13.16
Basic N	2.12	0.95	17.11	7.67	59.19	11.34
Nonbasic N	8.87	6.31	71.59	50.93	28.86	75.48

The casein used was not pure. In addition to considerable ether soluble matter it probably contained small amounts of nitrogen bodies other than casein. Pure casein from cow's milk contains 10.3 per cent of the nitrogen in the form of amids and 22.4 per cent as basic nitrogen compounds, while the above material yielded 12.43 per cent as amids and only 17.11 per cent as basic compounds.

The bacterial action was allowed to continue for three days, during which time 31.96 per cent of the total nitrogen was converted into ammonia. The amid nitrogen was reduced from 12.43 per cent to 8.88 per cent of the total, basic nitrogen from 17.11 per cent to 7.67 per cent, and nonbasic nitrogen from 71.59 per cent to 50.93 per cent. Expressed in percentages of decrease we find that 28.57 per cent of the amid nitrogen, 55.19 per cent of the basic, and 28.86 per cent of the nonbasic nitrogen were ammonified. The organic nitrogen remaining at the close of the experiment was composed of 13.16 per cent amid, 11.34 per cent basic, and 75.48 per cent nonbasic nitrogen compounds. Comparing these percentages with the composition of the original casein it will be seen that the basic nitrogen compounds were decomposed more rapidly than the amids or nonbasic nitrogen compounds.

DRIED BLOOD.

Nitrogen content of dried blood and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	6.11	....	45.19	....	....
Amid N	1.23	.67	9.09	4.95	45.53	9.04
Basic N	2.52	1.09	18.64	8.06	56.75	14.71
Nonbasic N	9.77	5.65	72.26	41.76	42.17	76.25

The dried blood underwent bacterial decomposition for seven days and 45.19 per cent of the nitrogen was ammonified. The amid nitrogen decreased from 9.09 per cent to 4.95 per cent of the total nitrogen, the basic nitrogen from 18.64 per cent to 8.06 per cent, and the nonbasic nitrogen from 72.26 per cent to 41.76 per cent. Calculating the percentages of decomposition in the different groups we find that 45.53 per cent of the amid nitrogen, 56.75 per cent of the basic nitrogen, and 42.17 per cent of the nonbasic nitrogen were ammonified. These data show that the basic diamino acids were decomposed more rapidly than the other groups. The organic nitrogen remaining after the bacterial action was composed of 9.04 per cent amid, 14.71 per cent basic, and 76.25 per cent nonbasic nitrogen compounds, as compared with 9.09 per cent amid, 18.64 per cent basic, and 72.26 per cent nonbasic nitrogen in the original dried blood.

SOY BEAN CAKE MEAL.

The bacterial decomposition of this material took place for five days, with the results as given below:

Nitrogen content of soy bean cake meal and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	4.10	....	49.52	....	....
Amid N	1.24	.69	14.97	8.33	43.06	16.51
Basic N	.76	.24	9.18	2.89	67.10	5.74
Nonbasic N	6.28	3.25	75.84	39.25	48.25	77.75

The above table shows that 49.52 per cent of the total nitrogen was ammonified. The amid nitrogen decreased from 14.97 per cent to 8.33 per cent of the total, the basic diamino acids from 9.18 per cent to 2.89 per cent, and the nonbasic nitrogen from 75.84 per cent to 39.25 per cent. Expressed in percentages of decomposition it is found that 43.06 per cent of the amid nitrogen, 67.10 per cent of the basic nitrogen, and 48.25 per cent of the nonbasic nitrogen were ammonified. The organic nitrogen remaining was composed of 16.51 per cent amid, 5.74 per cent basic, and 77.75 per cent nonbasic nitrogen compounds, as compared with 14.97 per cent amid, 9.18 per cent basic, and 75.84 per cent nonbasic nitrogen in the original material.

COTTONSEED MEAL.

Cottonseed meal was exposed to bacterial action for eight days, with the results shown in the following table:

Nitrogen content of cottonseed meal and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	1.45	....	28.43	....	....
Amid N	0.78	0.59	15.29	11.57	24.37	16.15
Basic N	.96	.32	18.83	6.27	66.67	8.77
Nonbasic N	3.36	2.74	65.88	53.72	15.48	75.07

The above data show that 28.43 per cent of the total nitrogen was ammonified. The amid nitrogen decreased from 15.29 per cent to 11.57 per cent of the total, the basic nitrogen from 18.83 per cent to 6.27 per cent, and the nonbasic nitrogen from 65.88 per cent to 53.72 per cent. Expressed in percentages of decomposition it was found that 24.37 per cent of the amid nitrogen, 66.67 per cent of the basic nitrogen, and 15.48 per cent of the nonbasic nitrogen were ammonified. Thus it is found that the basic diamino acids were ammonified more rapidly than the other groups.

LINSEED MEAL.

Linseed meal was subjected to bacterial action for seven days, with the following results:

Nitrogen content of linseed meal and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	1.99	....	39.80	....	....
Amid N	0.83	.64	16.60	12.80	22.89	21.26
Basic N	.62	.38	12.40	7.60	37.09	12.62
Nonbasic N	3.55	1.99	71.00	39.80	43.66	66.11

It will be seen from the above table that 39.80 per cent of the nitrogen was ammonified. The percentages of decomposition show that 22.89 per cent of the amid nitrogen, 37.09 per cent of the basic nitrogen, and 43.66 per cent of the nonbasic nitrogen were ammonified. The organic nitrogen remaining was composed of 21.26 per cent amid, 12.62 per cent basic, and 66.11 per cent nonbasic compounds, as compared with 16.60 per cent, 12.40 per cent, and 71 per cent, respectively, in the original material. Thus it is shown, in contrast

to the materials reported above, that the nonbasic monamino acids of linseed meal were decomposed more rapidly than the nitrogen compounds of other groups.

COCONUT MEAL.

Preliminary ammonification experiments with this material indicated that the nitrogen constituents would be decomposed more slowly than in the materials reported above. After incubating one week practically no ammonia was found. Consequently the decomposition was allowed to take place for 12 days, but even then only a small amount of ammonia was formed.

Nitrogen content of coconut meal and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	0.24	....	7.27	....	....
Amid N	0.38	.37	11.52	11.21	2.67	12.09
Basic N	.52	.41	15.76	12.43	21.15	13.39
Nonbasic N	2.40	2.28	72.72	69.09	5.00	74.51

It would seem that the carbohydrates and fats protected the nitrogen bodies from bacterial decomposition, since only 7.27 per cent of the total nitrogen was found as ammonia, and the absolute amounts of nitrogen in the different groups were only slightly different from those in the original material. But the magnitude of the experimental error was relatively too great to justify positive conclusions.

The data show, however, that a higher percentage of the ammonia was derived from the basic nitrogen group than in any of the previous experiments.

GLOBULIN FROM COTTONSEED MEAL.

The globulin was prepared from cottonseed meal by extraction with a 10 per cent solution of sodium chlorid, then precipitated by saturating the solution with ammonium sulphate, redissolved in sodium chlorid solution, and dialyzed. The product was washed with alcohol and ether, and dried in vacuum over sulphuric acid, but was still impure as shown by the nitrogen content. Ammonification continued for three days.

Nitrogen content of globulin from cottonseed meal and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	4.55	....	27.77	....	....
Amid N	1.80	1.04	10.99	6.35	42.22	8.81
Basic N	3.77	1.87	23.02	11.41	50.39	15.80
Nonbasic N	10.81	8.92	65.99	54.46	17.48	75.40

The nitrogen of the original material was composed of 10.99 per cent amid, 23.02 per cent basic, and 65.99 per cent nonbasic compounds. Pure globulin from cotton seed, on the other hand, contains 10.3 per cent amid, 30.6 per cent basic, and 59.1 per cent nonbasic nitrogen. It is possible that the low percentage of basic nitrogen and the correspondingly high percentage of nonbasic nitrogen found above were due to incomplete hydrolysis, as Osborne has pointed out that some vegetable proteins require continuous boiling for 24 hours for complete hydrolysis.

The above data show that 27.77 per cent of the nitrogen was converted into ammonia, and that 42.22 per cent of the amid, 50.39 per cent of the basic, and 17.48 per cent of the nonbasic nitrogen compounds were decomposed. The organic nitrogen remaining after bacterial action was composed of 8.81 per cent amid, 15.80 per cent basic, and 75.40 per cent nonbasic compounds. Comparing the above data with that obtained with the use of cottonseed meal, it is of special interest to note that globulin, when separated from the other nitrogen and nonnitrogenous constituents of cottonseed meal, undergoes bacterial decomposition in very much the same way as do the nitrogen compounds of cottonseed meal as a whole. In each instance the basic diamino acids were decomposed more rapidly than the other groups. The amids, however, were decomposed more rapidly in the globulin than in cottonseed meal.

ZEIN FROM MAIZE.

With the exception of linseed meal the basic diamino acids of the preceding materials were decomposed more rapidly than the amids or monamino acids. The basic nitrogen in these materials varied from 9.18 per cent to 23.02 per cent of the total nitrogen. In order to study the decomposition of a substance containing still less diamino nitrogen, zein was prepared from maize by alcoholic extraction. The product was not highly purified but the analysis shows that practically all the nitrogen was in the form of zein.

Nitrogen content of zein from maize and its bacterial decomposition products.

	Per cent of original material.		Per cent of total N.		Per cent of groups decomposed.	Per cent of organic N after bacterial action.
	Before.	After.	Before.	After.		
Ammonia N	....	0.98	....	6.98	....	....
Amid N	2.75	2.21	19.60	15.75	19.64	16.93
Basic N	.43	.38	3.06	2.78	11.63	2.91
Nonbasic N	10.85	10.46	77.33	74.55	3.59	80.15

Bacterial action was allowed to continue for three days, but, as shown in the table, only small amounts of ammonia were formed. The zein as prepared was in a tough, horny condition, and was consequently difficult to pulverize, which probably accounts in part for the low yields of ammonia. The data show that the basic diamino acids were only decomposed to a slight extent; but in this case the amids were most markedly decomposed. The decreases in amid nitrogen as determined, however, may not have been entirely due to ammonification, since any amid compounds that were split off by the bacteria, but not ammonified, would have been decomposed and determined as ammonia along with that actually formed by the bacteria.

As shown above, a large portion of the nitrogen in the materials used was not ammonified, or at least did not occur at any one time as ammonia; neither was the yield of ammonia from casein in the experiments with soil materially increased by prolonging the time of decomposition beyond four days. But the cessation of ammonification was not due to the accumulation of poisonous by-products, since the second and third grams, added after the ammonification of one gram had come to a stop, were each ammonified to a slightly greater extent than the first gram. It seems probable, therefore, that a part of the organic nitrogen in the materials used is more resistant to ammonification than others. It should also be remembered that putrefactive decomposition usually takes place to some extent in the ordinary ammonification experiment, which probably results in the formation of the aromatic protein cleavage products, tyrosin, phenylalanin, and tryptophane at first; later these are decomposed into indol and skatol, rather than being immediately converted into ammonia. It seems also that a portion of the nitrogen was assimilated by the organisms present, but whether the assimilation of ammonia or organic forms took place can not be definitely stated. The latter seems the more probable. In either case it is reasonably certain that synthesis as well as decomposition plays a considerable part in the chemistry of soil organic nitrogen.

Finally the basic diamino nitrogen of organic materials is ammonified, or otherwise loses its identity as such more rapidly than the

amids and monamino acids. This phase of the chemistry of bacterial action is in harmony, therefore, with the indications given by previous study on the organic nitrogen of soils.

SUMMARY.

(1) The ammonification of casein in silica sand was much more rapid during the first two days than that of dried blood, soy bean cake meal, cottonseed meal, or linseed meal, while soy bean cake meal was second in the order of decomposition. Later loss of ammonia by evaporation reduced the concentration of ammonia, thus making it impossible to compare the rates of decomposition.

(2) During the first two days the rate of ammonification in soil was similar to that in sand, and a much higher percentage of the total nitrogen in casein was ammonified than of the other materials. On the ninth day 50.2 per cent of the casein nitrogen, 42.4 per cent in dried blood, 40.9 per cent in soy bean cake meal, 27.1 per cent in cottonseed meal, and 26 per cent in linseed meal had been ammonified.

(3) When equal amounts of nitrogen were added, casein still underwent more rapid ammonification during the first two days than the other materials, and cottonseed meal and soy bean cake meal were more completely ammonified than dried blood or linseed meal. Later the yield of ammonia from dried blood exceeded that from cottonseed meal. During the nine days of the experiment 56.9 per cent of the nitrogen in casein, 49.3 per cent in dried blood, 48.7 per cent in soy bean cake meal, 32 per cent in cottonseed meal, and 34.6 per cent in linseed meal were ammonified.

(4) Under anaerobic conditions all of the materials were ammonified very slowly during the first two days. Later the casein was converted into ammonia approximately to the same extent as under aerobic conditions, but the other materials were decomposed much less vigorously.

(5) With equal amounts of both nitrogen and nonnitrogenous matter present the final yields of ammonia from the different materials, with the exception of dried blood, agreed closely, but the initial decomposition of casein was still much more active than the other substances. The yield of ammonia from casein on the ninth day was only 31.4 per cent as compared with 56.9 per cent in the absence of starch, and the ammonification of dried blood was reduced from 49.3 per cent to 18.9 per cent. It has been suggested that the ammonifying organisms are able to utilize carbohydrates to some extent as sources of energy. If so, smaller amounts of ammonia would consequently be split off from proteins in the presence of carbohydrates. Hence the carbon-nitrogen ratio would materially affect the actual formation of ammonia in soils.

(6) When the amounts of casein were varied, other conditions remaining constant, the yields of ammonia in four days increased as the amounts of casein present increased; 48.4 per cent of the total nitrogen in 0.2 gram was ammonified, 57 per cent in 1 gram, 60.9 per cent in 2 grams, and 65.9 per cent in 3 grams. It seems probable that decreasing percentages of the total nitrogen were assimilated by the organisms present as the amounts present increased, but there are probably other factors of a chemical and biological nature involved.

(7) The yield of ammonia from casein was not materially increased by extending the incubation period beyond four days, and the decomposition of the second and third gram, added after one gram had been acted upon four and eight days, respectively, was slightly more vigorous than that of the first gram. In each instance approximately 60 per cent of the total nitrogen was found as ammonia. These facts, taken in connection with the above, indicate that the incomplete ammonification was not due to the inhibitory effect of the decomposition products, but rather that a part of the nitrogen of casein is extremely resistant to ammonification. It is also possible that a large part of the remaining nitrogen was assimilated by the bacteria.

(8) Casein when mixed with silica sand or in solution was completely hydrolyzed by the action of bacteria in seven days. In the former instance, 64.2 per cent of the nitrogen was ammonified and in the latter 59.53 per cent. In solution the rate of hydrolysis exceeded that of ammonification, but the latter was not so active during the first five days as when mixed with soil (see Series IV).

(9) The determination of the different groups of nitrogen compounds before and after bacterial action in casein, dried blood, soy bean cake meal, cottonseed meal, linseed meal, coconut meal, globulin from cottonseed meal and zein from maize shows that, with the exception of linseed meal and zein, the basic diamino acid nitrogen was converted into ammonia more rapidly than the nitrogen of other groups. With casein, soy bean cake meal, and cottonseed meal the more rapid ammonification of the basic nitrogen was especially noticeable. When this fact and the above are considered in connection with a comparison of the organic nitrogen of soils and vegetable proteins, it becomes apparent that all portions of the organic nitrogen in the different materials used as fertilizers and green manures are not equally susceptible to ammonification. It is evident, therefore, that chemical factors inherent in the nitrogen compounds themselves predetermine the availability to some degree. Further investigation, including a study of the decomposition of individual amino acids and acid amids, is being made.

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